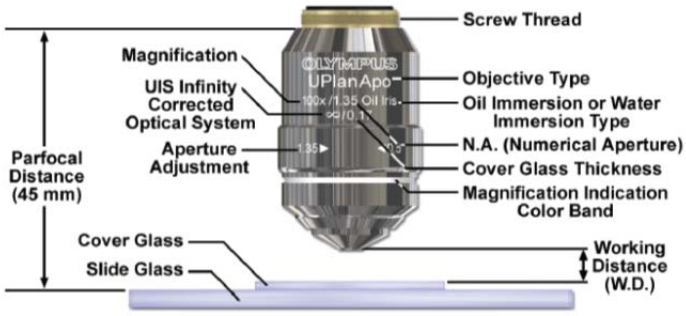
Dante (Microscope) & Beatrice (Guide) Orth Lab

Olympus IX81 Widefield Microscope

User Guide

v. 1.2 (11/2014)

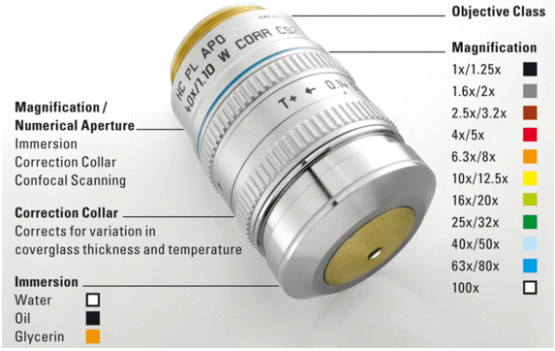


Objectives

4x/0.13NA UPLFLN Semi-Apo
10x/0.4NA PH UPLAPO Plan Apo
20x/0.8NA PH UPLAPO Plan Apo
40x/0.75NA PH UPLFLN Semi-Apo

100x/1.4NA oil UPLSAPO Super Apo

Standard coverslip thickness 0.17 (#1.5 coverslip)



Light Source & Filter Sets

Prior Lumen 200 Pro - 200 watt metal halide lamp

Label	Excitation	Dichroic	Emission
QD 360	(1) 350/50	(2) T400LP	(3) 500LP ?
BODIPY	(3) 570/20	(4) 585LP & 620/60 (Chroma 41034)	
DAPI	(1) 350/50	(2) T400LP	(2) 460/50
CFP	(1) 438/24 & 458LP & 483/32 (Semrock 2432C)		
FITC/GFP	(2) 470/40	(3) 495LP & 525/50 (Chroma 49002)	
YFP	(5) 500/25 & 525LP & 545/35 (Omega XF104-2)		
Texas Red	(4) 560/40	(4) 585LP & 620/60 (Chroma 41034)	
Cy5/AF647	(6) 628/40 & 660LP & 692/40 (Semrock Brightline 4040A)		
Brightfield	(5) open	(2) T400LP	(5) open

Camera

Hamamatsu Orca R2 CCD camera (C10600-10B; 6.45 µm x 6.45 µm pixel)

Contents:

Quick Guide:

1) Turn on components in order.
2) Log into Windows with your IdentiKey and start Slidebook 6 software.
3) Put sample on scope coverslip down. If using oil immersion, do not switch back to air objective. No oil on air objectives!
4) Find and focus your sample through the oculars.
5) Acquire images, timelapses, Z-series, etc.
6) Save and copy your data to the E:\ drive. The LMCF is not responsible for long-term data storage. See Appendix A for information on how to transfer data to MCDB Dept Server.
7) Lower stage away from sample, and remove sample from stage. If you used the environmental chamber, properly disconnect and shut it down. Properly clean oil off of any immersion objectives you may have used with lens paper. If you are at all unsure of this process, ask for help!
8) Leave the microscope on a low power objective for next user.
9) Close Slidebook.
10) Log use in Excel sheet, save and close.

➔ **If someone is signed up within the next hour (Check the MCDBCal!):**

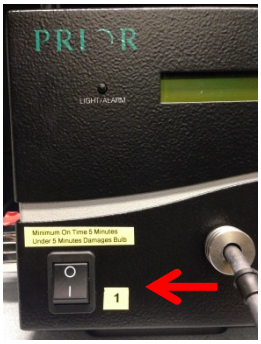
11) Log off Windows and leave the system on

➔ **If no one is signed up within an hour or you are the last of the day:**

11) Shut down system in reverse numerical order: PC, Hamamatsu camera controller, Olympus microscope controller, Prior Proscan III stage controller, Prior Lumen 200 Pro light source.

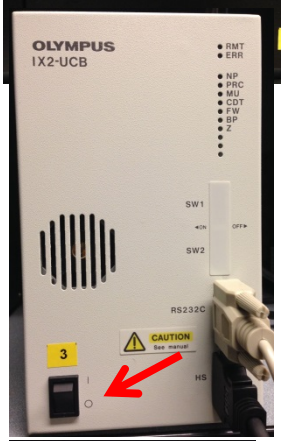
Starting Up the System:

1) Turn on the Prior Lumen 200 Pro epi-illumination light source. If this was turned off within the last 30 minutes – WAIT until the lamp has cooled before turning it back on.

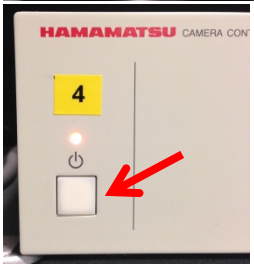


2) Turn on the Prior Proscan III stage controller. The power switch is in the back, bottom right corner.

3) Turn on the Olympus IX2-UCB microscope controller power supply.



4) Turn on the Hamamatsu Orca R2 camera controller (you may have to press and hold for a little bit to have the light turn green).



5) Turn on the PC and login with your IdentiKey.

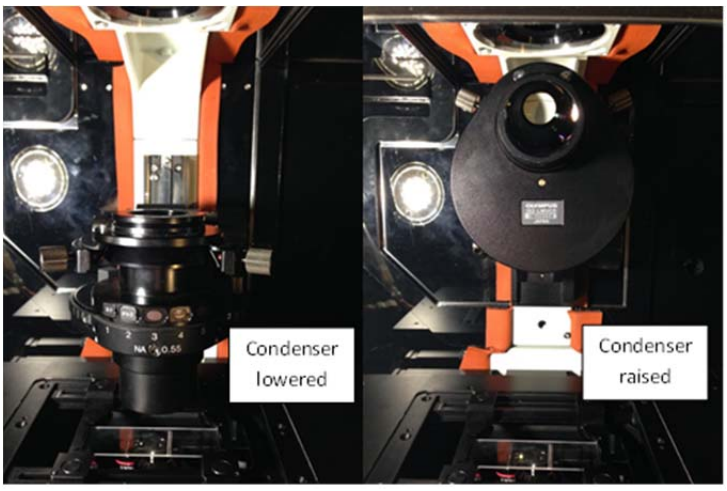
Environmental Control System:

<u>Before using this – please contact a member of the Orth lab to obtain permission and training.</u>

If you are going to be keeping a culture at an elevated temperature on the scope stage, turn on the Environmental Control Unit with at least an hour's lead time; it takes a while to bring the environmental stage (which will contain the tissue culture dish with its glass bottom for specimen viewing) up to temperature. If you are using this, connect up all the leads to the stage (power, control, and gas), and turn on the gas as the control unit requires about 30 min to bring the system to 5% CO_2. Leave the environmental stage outside of the microscope until it equilibrates to make sure the system does not leak into the microscope. Place an empty dish in the insert to partially seal the system and help it equilibrate faster. Once it is stable and at temp/CO_2, place the unit on the microscope stage.

Operating the microscope:

<u>The condenser</u>: This is a long working-distance device, not high resolution (NA = 0.55). It contains on phase annulus, appropriate for "Phase 2" lenses. This is in position 2 on the phase turret. All other positions on the turret are currently "bright-field". The condenser can flip up, away from the stage, making it much easier to insert or modify the specimen. BE SURE to lower it back into position, once the specimen has been inserted if you need trans-illumination (bright-field). When the condenser is lowered, its vertical position can be adjusted with a rack and pinion knob, but be careful not to lower it so far that it touches the top window of the environmental stage. Have it as low as it will go without touching.



<u>Objectives</u>: The microscope currently has 4x/0.13NA UPLFLN Semi-Apo and 100x/1.4NA oil UPLSAPO Super Apo non-phase objectives and 10x/0.4NA PH UPLAPO Plan Apo, 20x/0.8NA PH UPLAPO Plan Apo, and 40x/0.75NA PH UPLFLN Semi-Apo phase objectives. Trying for high

resolution imaging with trans-illumination when using a long working distance condenser is a waste of time. Fluorescence imaging should, however, be OK as this does not use the condenser (objective will function as condenser). On the scope table beside the scope there is a tab box for changing objectives electronically. This can also be done from the computer. Do not change objectives manually (i.e., don't rotate the turret with your hands).

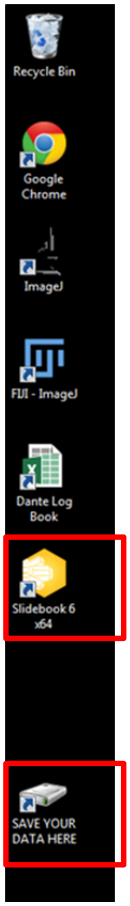
Filters: The scope is currently set up with QD360 (similar to DAPI but for quantum dots), DAPI (which works for Hoechst DNA stains too), CFP, FITC (which works for GFP), YFP, Texas Red (which works for RFP and mCherry), and Cy5 (which works for Alexa 647). There is also a blank filter position, called "brightfield" which has no filters. Filters are changed from the computer (see below). The exact wavelengths and configurations for illumination are outlined on the cover page.

Objective focus (stage position in Z): There is a knob on both sides of the microscope stand which can be either fine or coarse focus, depending on which tab is pushed (F/C is a toggle). The "escape tab" will take the stage to a pre-set lowered position. The speed with which the focus changes with each turn of the focus knob depends on the objective in place and will move more slowly for higher magnification (shorter working distance) lenses. However, be careful not to push the objective up against the slide.

Stage position in XY: This is controlled by the joystick: direction stick is moved is the direction the stage will appear to move. Displacement of stick controls speed of stage movement. There are tabs on the joystick box which don't do anything.

Camera: The digital camera (Hamamatsu Orca R2, (C10600-10B; 6.45 μm x 6.45 μm pixel) is on the left of the scope as you look at it, and there is no need to touch it.

Operating the Computer:

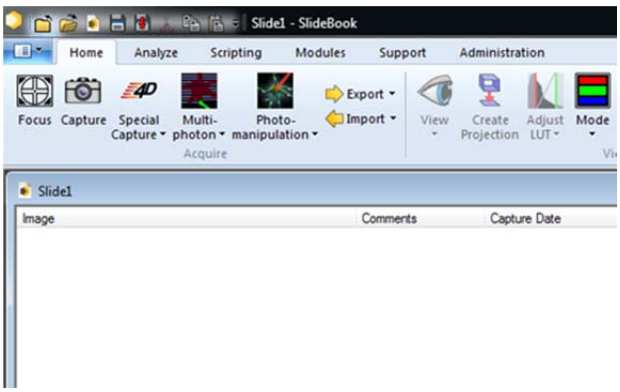


Login with your Identikey and password.

Make a directory for your own work on the E:\ (Data) drive within a folder with your lab name (so for example: E:\McIntosh lab\JRM). There is a shortcut on the desktop called "SAVE YOUR DATA HERE." Do not save your data to the C:\ drive as this drive is not large enough for image storage, and filling the C:\ drive will lead to system instability (=crashing = bad).

Open Slidebook 6 by double clicking on its icon on the desktop. If at any point while using Slidebook you need help/explanation of a particular window or option press F1 with that window open and the Help will open to the page pertaining to that function or window.

Slidebook will open with a new "slide", which is the sub-directory in which your work from the current session will be saved. Save this slide to your own directory. This is necessary to activate several of the Slidebook icons.

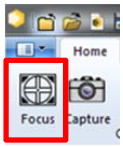


At the very top of the Slidebook widow, there is a set of icons. Each of these performs an action with respect to your new "Slide". Their functions are displayed by a tooltip when the cursor is on them.

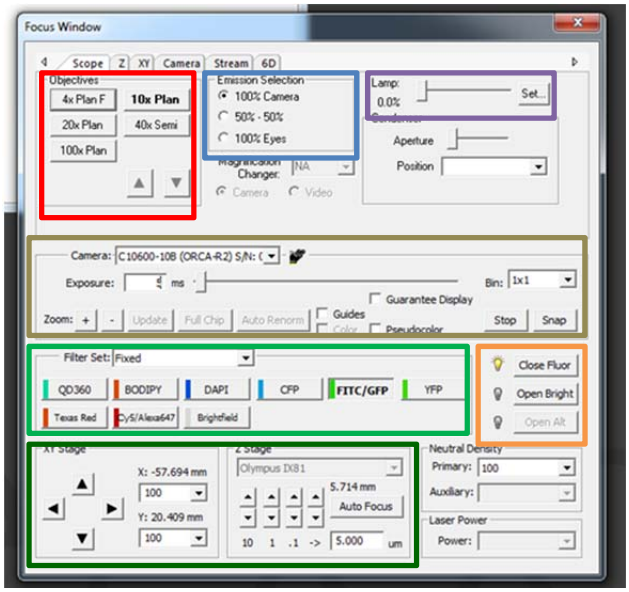
Down one row, the left-most icon is for a drop-down menu that gives options similar to the ones across the top of the screen, including the options to save and open files. The next position is Home, which is the place to be for operating the scope. All the other options are for additional Slidebook functions, e.g., analysis, which will not be covered here in detail.

The Focus Window:

When Home is clicked, there is a set of icons for scope operation. The first is Focus, and this opens a window that controls many aspects of microscope function.



The Focus window has its own set of tabs across the top, and you will start with Scope, which controls the choice of **objective** and **filter**, as well as whether the light from the scope **should go to the camera or the eyepieces**. It also controls the **shutters** that protect the sample from either the trans-illumination (Open/Shut Bright) or epi-illumination (Open/Shut Fluor). Note that when the tab says, "Open", it means that the shutter is closed and clicking it will accomplish the actions shown in the tab. Vice versa for shutting. The little lamp icon will appear as a yellow light when the shutter is open/light is on.



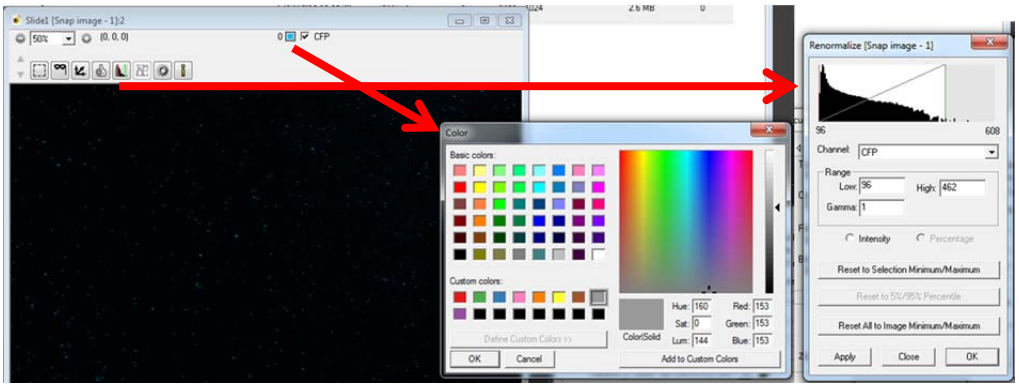
There is a **slider, called "Lamp"** that controls the brightness of the trans-illuminator. If you want to use trans-illumination, make sure that the condenser is in the down position and move the lamp slider to about 20% (you can adjust as needed, but this is a good starting position.) Then click on the "Brightfield" filter option and "Open Bright".

If there were a choice of cameras, you would make it here, and you can set both the **exposure time for the camera** and the binning of the image. Choose the conditions that will minimize photobleaching. For dim samples, or where resolution is not so important, one can also increase the binning. Snap will show a view of the image one is getting. At the lower left of the window are arrow-heads that will **move the stage**, just the way the joystick does, and there are windows that allow a choice of the size of the steps that clicking the arrows will achieve. There are also controls for **Z and for auto-focus**; more on that below.

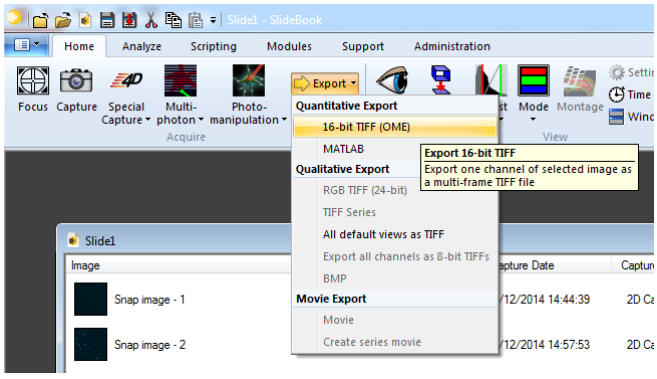
The tabs for "magnification changer", "condenser", "laser power", and "neutral density" are currently not set up, as these cannot be controlled electronically or are not available on this system.

Focus Window – Looking at your sample and taking a Snap:

Once the appropriate objective and filter have been selected, one can use Open Fluor or Open Bright to look at the sample. Select "100% Eye" to be able to view the sample through the eyepieces. "100% Camera" will send the signal to the camera, and "Live" will display the image on the screen and continuously exposed to illumination. While viewing live, one can bin 2x2 with no loss in visible detail and with some increase in brightness without any increase in exposure of the cell to light. "Snap" will take a picture of it, opening the shutter for the pre-set amount of time (the exposure time). The color shown in the display is a bogus, pseudo-color, and it not part of a save image file. You can adjust the pseudo-color and look-up table (LUT) scaling as desired.



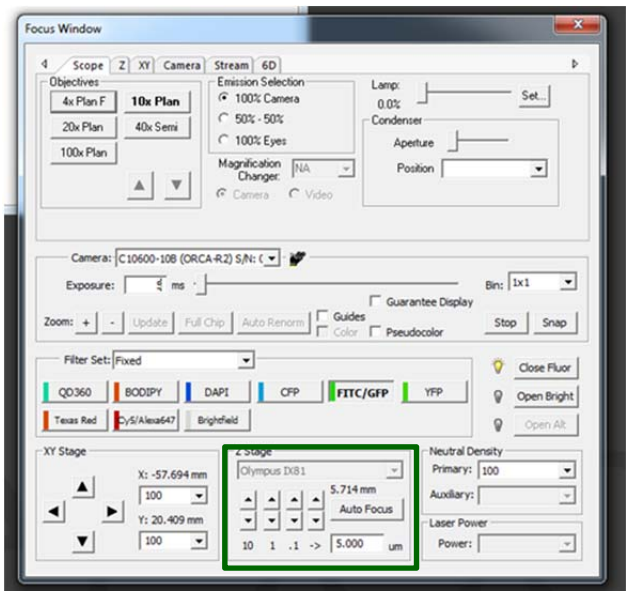
"Snap" saves the image in a Slidebook proprietary format. It will go into the open "Slide" with an identifying name, but it will not be available for view on other computers (that don't have Slidebook). To get really useful images, one will want to export the images as TIFFs. It is highly recommended that you save both the proprietary "Slide" file as well as exporting as TIFFs, because the "Slide" file represents your raw data with all the associated metadata (info on acquisition parameters). Avoid saving files with lossy compression types (e.g., jpeg).



Focus Window – Autofocus:

The "Autofocus" function is very useful in a time or space series, because focus is not stable, and this function can correct for it. When this button is clicked, there are options for which protocol to use. In phase optics (trans-illumination), the Gaussian Derivative works best; in

fluorescence (epi-illumination), use “Spectral 2D”. Set the total search range to define how big a range of Z the scope will use in trying to find the best value. It is usually appropriate to set this for somewhere between 5 and 10 µm. Now, readjust the number of steps and this will set the step-size. A good setup uses 0.2 or 0.5 µm steps over a 5 µm range. The Peak Delta Threshold is the sensitivity of the algorithm. If specimen contrast is high, as in phase imaging, 0.2 is a good value. For lower contrast (bad phase contrast or weak fluorescence), use 0.6.

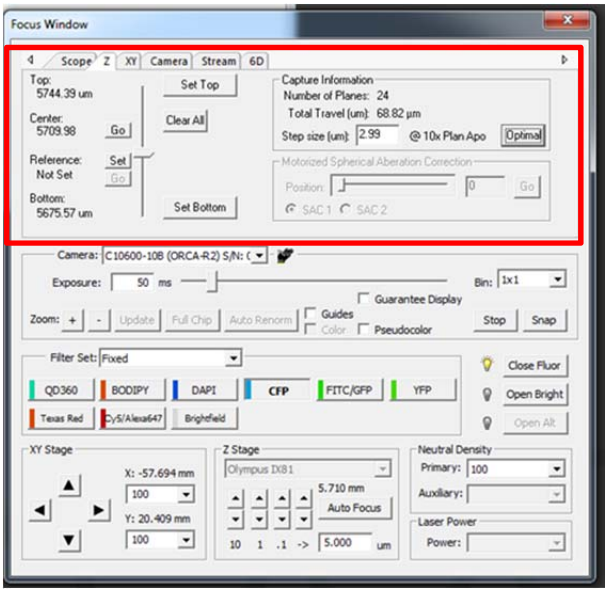


One can test the effectiveness of the autofocus when the sample is in the first location one wants to image. Selecting “autofocus” from the auto focus window is a way of checking the auto-focus settings. Best Z should indicate the position if the program finds a correct plane of focus. If Best Z says “At Edge” it means the program thinks the correct plane of focus is outside of the search range. If Best Z says “No Gradient” the program does not think an adequate plane of focus exists.

Note that once these parameters have been set, one MUST ALSO GO TO “Advanced” in the CAPTURE window and click Autofocus there in order to have it work during a capture series (clarified later).

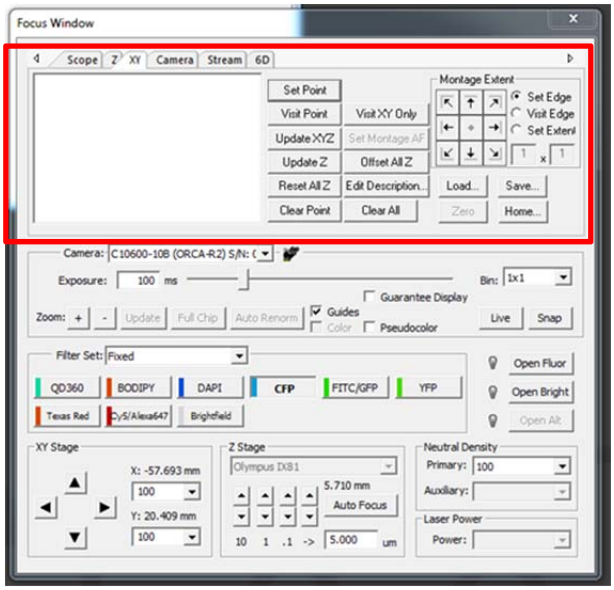
Focus Window – Z Tab:

This is the place from which you can set up a Z-stack for 3D (or 4D) imaging. After pushing the “Z” tab at the top of the Focus widow, one can set up a “Z-stack”, i.e., a set of instructions that will tell the scope to take a set of images at one XY location at the pre-set values of Z. There are a couple of ways to implement this: Set focus by hand and use it to “set top” (then push that button) and “set bottom” and then set step size. This will define the number of image planes to be captured. Alternatively, one can set top and bottom and number of planes; this will set step size. The fewer the steps, the less



photobleaching, but more steps yields higher resolution in Z up to a point. The point-spread in Z is about 1.5 – 3x the resolution in XY, thus very small step sizes will not yield additional information.

Focus Window – XY Tab:

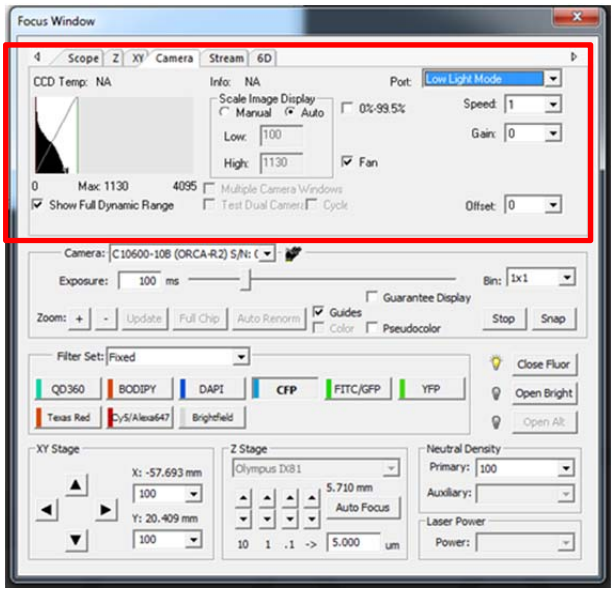


This is the place from which you can choose multiple locations on your sample to visit in sequence. This allows making a time-lapse (TL) movie of multiple locations at the same time, a huge time saver. After pushing the "XY" tab at the top of the Focus window, one can choose multiple locations at with to do an imaging job, either at one focal plane or as a stack, when a Z-stack has been defined. To make these choices, one can use the joystick or computer control to motor around the sample, choosing regions of interest. When you have one, and it is in good focus, click "Save XYZ" and the computer then knows to go to just that point. Almost any number of locations can be picked, but be aware that the number of images taken (in Z, over time, and at multiple XY locations) will soon add up to a big file! Once a set of sample locations has been chosen, once can click "save" on this window, and these addresses will be saved with a filename that you give for use at a future time. Whether or not you save them, the computer can use them, both to control automatic operation and to let you visit these locations and make sure they are what you want. When at a location, you can adjust X,Y, or Z and then click "Update Z" or "XY" and the new addresses will be stored. Stored addresses can be erased, one by one or all together, and replaced if desired. When the stage is motoring around, it will use the XYZ given as the start for a preset autofocus operation.

One can also set up the acquisition of a montage image by either defining the corners of the area to be acquired (go to that position and then click the corner with "Set Edge" selected). You must define at least two diagonal corners that use the same Z position, or three corners for slope interpolation. Slope interpolation will adjust the Z position along a Z plane that fits through all three positions. When you set up the capture, be sure to select Advanced → Autofocus → Autofocus interpolate best Z start position (clarified later). To acquire a montage around your current position, choose "Set Extent", choose how many fields of view to acquire

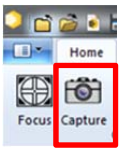
(e.g., 2x2 will acquire 4 images total) and click the center dot. If you want your current position to be the top left of the montage, click the top left corner button instead of the dot.

Focus Window – Camera Tab:



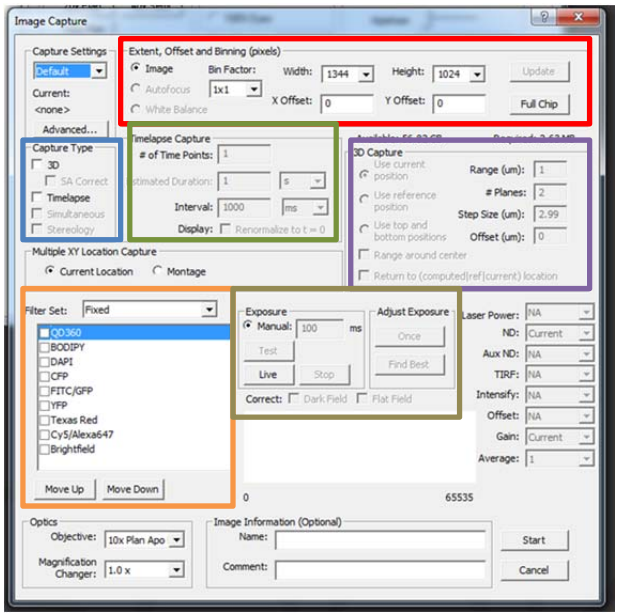
The next tab across the top of the focus window is Camera. This will display a histogram of the pixel intensities and allow the choice of manual or auto-scaling of image display. At the upper right there is a window saying “Low Light Mode”; in general this is preferable to the alternative “High Light Mode”. Speed should be 1 and gain 0.

The Capture Window:



The next tab across the top of the screen in Home is Capture. This opens a window that does what its name suggests. Here you can set up montages, 3D, and multiple locations for imaging.

Upper left: Capture Settings: leave it at Default.



Extent, Offset and Binning (pixels): Have “Image” clicked, leave Bin Factor at 1, unless you want to get more light into a single pixel, in which case, choose 2. Note that this will result in larger pixels and loss of spatial resolution (do you need to make very precise localization measurements?). Width is at 1344, Height at 1024, X and Y offsets at 0; this is at full chip, so that tab does nothing. If you have altered size, the full chip tab will bring you back to this maximum.

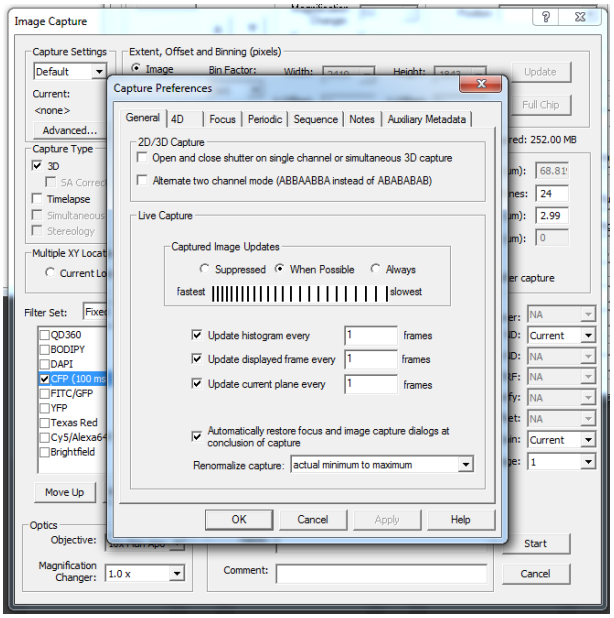
Capture Type: select either 3D, time lapse, or both. When these boxes are ticked, the windows just to the right for setting up each function will become active. The **Timelapse capture** window allows you to set conditions for that round of image acquisition. Do click on Display, Renormalize to t = 0. The 3D Capture Window will display the information you input in the Focus Window (see above).

Filter Set: something must be clicked here, or the scope won't operate. Make sure you click the filters you want to use. The images will be taken in order from the top, so if you have clicked several and want a particular order (e.g., DAPI last to minimize bleaching of the other fluors), you can order them by moving specific filters up or down. Note that the window above this says "Fixed". The microscope setup only contains one configuration of filters (all available).

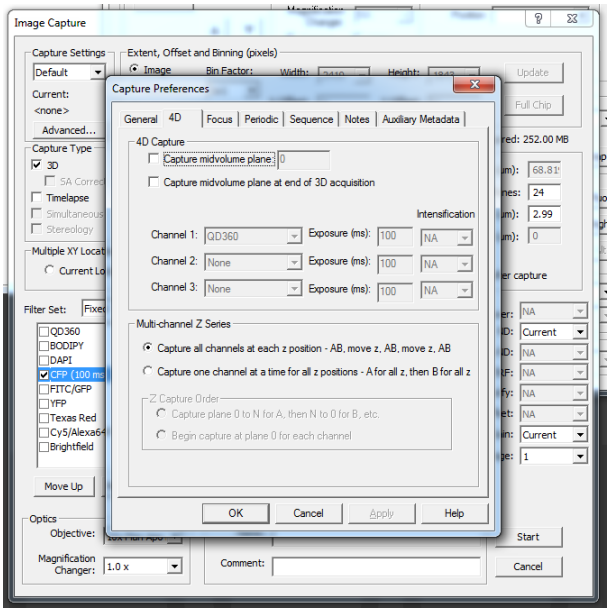
Exposure: Here, you can also set the exposure or click "find best". Under manual one can set a different exposure for each filter set, which can be very important. The window to the right of this (Laser power, etc.) is not operational. You can also choose your objective from here. (Magnification Changer doesn't work). You can also name your file and enter metadata as comment. "Start" will initiate the program you have specified.

The Capture Window – Advanced Options:

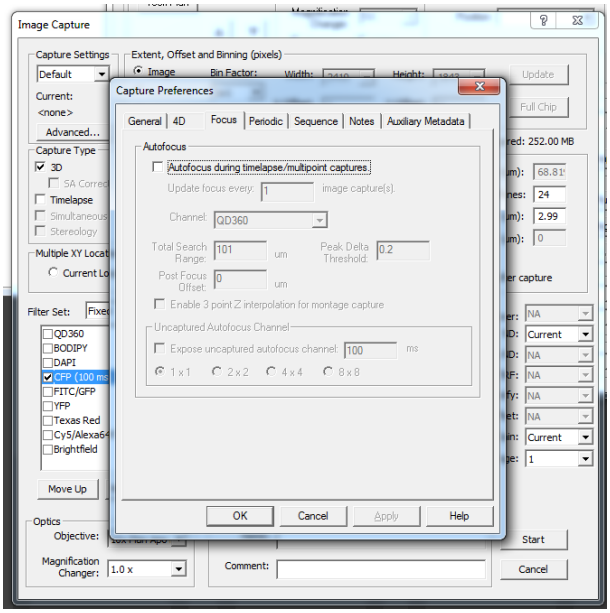
The Advanced button (top left) allows you to specify details of capture preference. In the **"General" tab** you can choose the order in which two channels are used: ABABAB, etc., or ABBAABBA, etc. This will affect the number of moves the scope has to make to follow your instructions. Camera image updates will control what you see on the monitor while the program is in action: updating when possible is often the best. This is a good balance between forcing the system to always update (even when memory may be maxed out) or never updating.



The **“4D” tab** again offers specific choices for a 4D acquisition.



The **“Focus” tab** allows you to choose when autofocus is done. Note, as mentioned above: THIS MUST BE CLICKED TO HAVE AUTOFOCUS WORK. Its conditions will be the ones set in the Focus window, but this tab is an essential part of having it work. The other tabs are somewhat self-explanatory, allowing you to add metadata, etc.

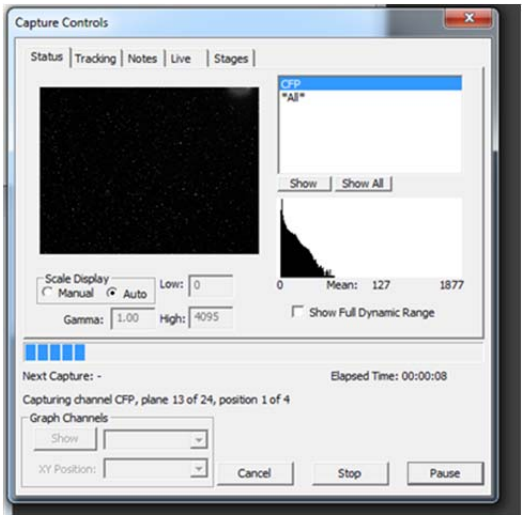


The Capture Window – Start the Acquisition:

After setting all the parameters and saving the slide in your lab folder, click the Start button in the bottom right to start the 2D/3D/4D image acquisition.

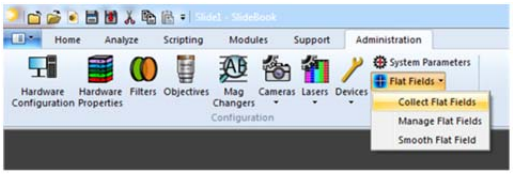
When collecting a time lapse series: The stage will visit the chosen locations that capture images with the filters and illumination one has chosen, with or without autofocus. To minimize drift in XY, do let the stage equilibrate at temperature for about ½ hour before

starting. Once capture has been initiated, a new, "Capture" widow appears, and it will describe what's going on (time until next capture set, time remaining in the whole program). One can stop the acquisition from this window, but that really does stop it, and one will have to restart. One can pause, and this is very useful, because now one can initiate a check on locations and focus at those locations, updating X,Y, and/or Z. The new locations will be saved automatically. If one is quick, this can all be done in one interval between image captures. Press the "continue" tab, and the TL program will continue without a break.

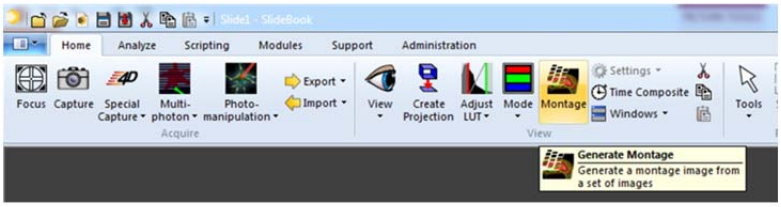


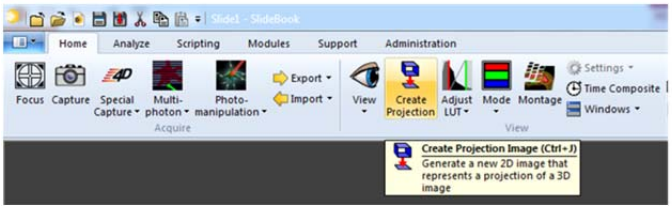
Other Features:

- When building a montage, acquire and apply a flat field correction to reduce the effect of uneven illumination in your final image.



- Create a projection of your 3D Z-series data.





When you're finished:

1) Save and copy your data to the E:\ drive. The LMCF is not responsible for long-term data storage. See Appendix A for information on how to transfer data to Puli.

2) Lower stage away from sample, and remove sample from stage. If you used the environmental chamber, properly disconnect and shut it down. Properly clean oil off of any immersion objectives you may have used with lens paper. If you are at all unsure of this process, ask for help!

3) Leave the microscope on a low power objective for next user.

4) Close Slidebook.

5) Log use in Excel sheet, save and close.

➔ **If someone is signed up within the next hour (Check the MCDBCal!):**

6) Log off Windows and leave the system on.

➔ **If no one is signed up within an hour or you are the last of the day:**

6) Shut down system in reverse numerical order: PC, Hamamatsu camera controller, Olympus microscope controller, Prior Proscan III stage controller, Prior Lumen 200 Pro light source.

APPENDIX A – Saving to your lab's folder on Collie (MCDB Dept Server):

➔ **Questions regarding MCDB Server should be directed to Erik Hedl.**

1) Map the network drive. Right click on Computer icon on desktop or Computer in Start menu. Click on "Map network drive…"

2) Drive letter should be Z: and the folder should be \\collie.int.colorado.edu\<your lab name> (for example \\collie.int.colorado.edu\OlwinLab).
   If your login for Collie is not your IdentiKey, check the box "connect using different credentials."

3) Click finish.

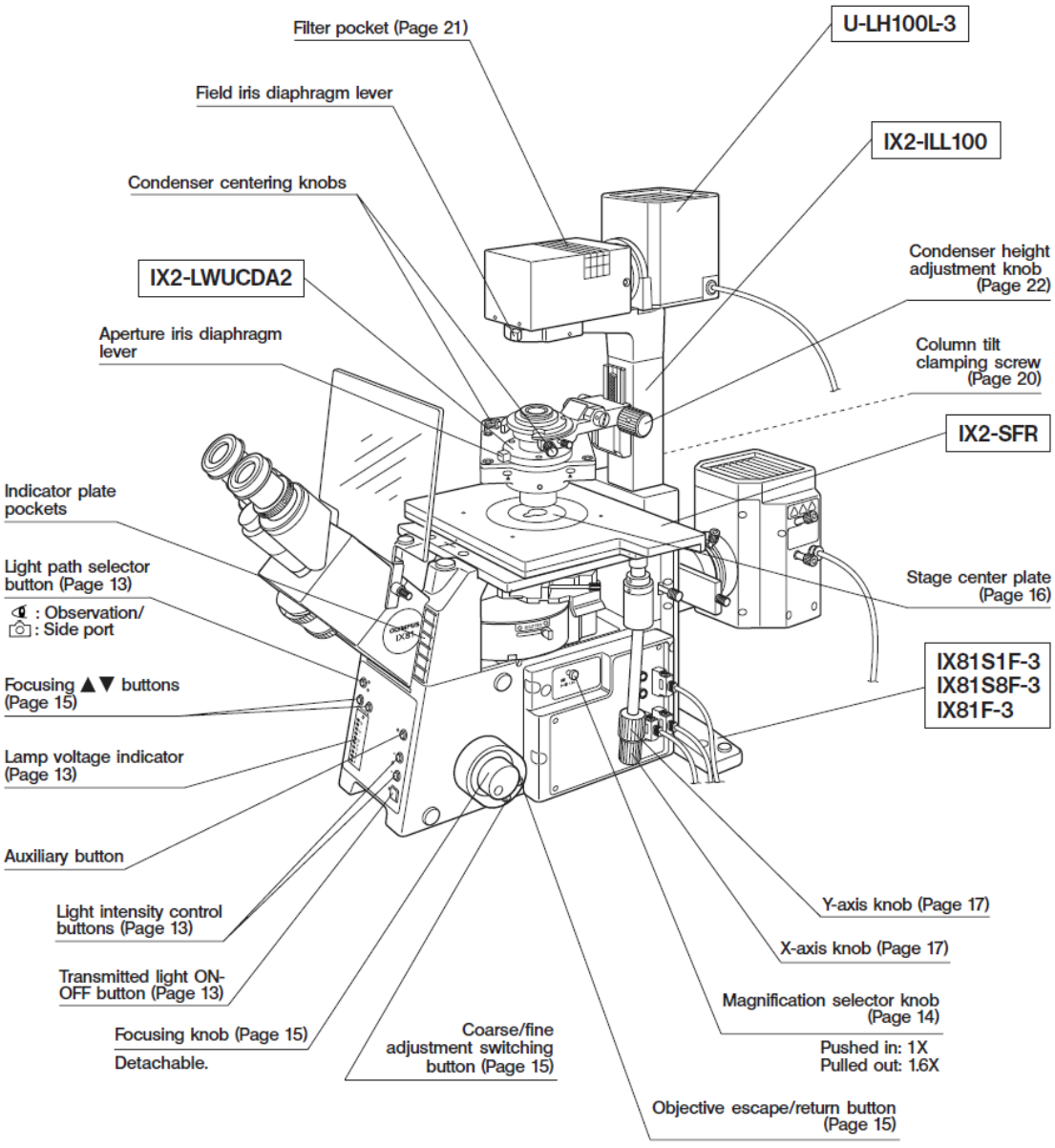
4) If you selected "connect using different credentials," enter your username and password.

5) Click Ok and the drive should now appear. Copy over your files. If you have a lot of files, be sure to allow time for the transfer. Under ideal conditions you will be able to copy close to 42 gigabytes per hour, don't count on ideal conditions. Also, it is safer to copy (not move) your files, then delete after the copy has completed.

➔ You cannot, must not, should not reserve time on the microscopes in MCDB Cal to copy data – reservations are for imaging only.
➔ The LMCF PCs are not long-term data storage places and we are not responsible for lost data.
➔ Do not forget to always safely store and backup your raw data – this represents the ground truth of what you acquired that day and has all the associated metadata. Many journals are now requiring that you submit raw data with your manuscript.

APPENDIX B – Parts of the microscope:

(not all these components are present on this microscope)



APPENDIX C – Factors that Affect Quality of Digital Images:

The goal of image acquisition is to acquire a quantitative image that is as close to the fundamental limits of resolution as you can achieve. This requires good signal to noise ratio (SNR). You want your signal to be visible above the background, which is affected by the noise level. Noise causes variations in intensity values and can come from a variety of sources and include: Poisson noise (shot noise, due to photon flux, less impact as signal increases), Read noise (errors as camera chip is read, independent of exposure time), and Dark noise (heat causes spurious electrons to pop, builds with exposure time). If your signal of interest is close to the background level and you have high noise, you will not be able to accurately resolve your signal within the noise variations.

To increase signal: use a #1.5 coverslip, clean coverslip and slide, mount as close to coverslip as possible, use camera binning

To decrease noise: optimize sample labelling protocol to maximize signal, use shorter exposure times

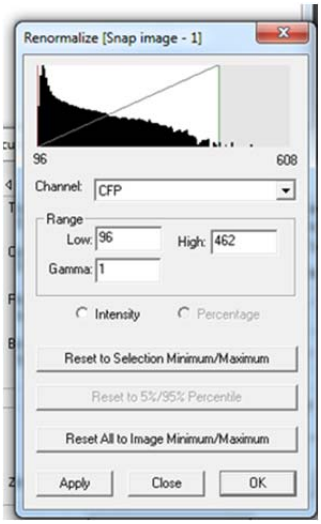
To decrease background: clean coverslips, optimize sample labelling protocol, dark room, close field diaphragm

1) **Exposure time**

The exposure time should be as fast/short as possible to obtain a good SNR image. Longer exposure times will lead to increased photobleaching, and if you want to acquire a thick Z-series (many planes) or are imaging live cells, don't set exposure time too high. For live cells, photobleaching and phototoxicity are more important considerations and exposure times should be carefully considered.

2) **Bit Depth / Autoscaling**

Slidebook by default will autoscale the image you acquire to show min-max range. It is imperative that your image not contain under- or over-saturated pixels since you will lose information regarding intensity distributions (e.g., can't tell the difference between a spot with intensity 5,000 or 10,000 – they both show up as 4,096 in an 12-bit image) or subtleties in structure (e.g., thin or thick membrane might look the same). You can adjust the LUT of the image to display a defined range or the full dynamic range.



3) **Binning**

Binning is the combining together or pooling or adjacent pixels into a single, larger pixel. For example, 2x2 binning will combine

a total of 4 pixels (2 per side) into a single bigger square pixel. This has the benefit of producing a brighter pixel (better SNR) at the same exposure time at the cost of reducing resolution. For live samples binning can be especially useful to allow for acquisition of a greater number of images (Z-series or timelapse) with less photobleaching and phototoxicity.